

ATTACHMENT II – PROTOCOL

Ecolab
Study Identification Number 1900080

REGULATED PESTICIDE EFFICACY STUDY PROTOCOL

STUDY TITLE: Synergex Efficacy against Food Contact Surface Associated Biofilm

EPA REG. NO.: 1677-250

ECOLAB GLP STUDY NUMBER: 1900080

PROPOSED STUDY INITIATION/COMPLETION DATES

Initiation	August 27, 2019
Completion	October 18, 2019

DESCRIPTION OF STUDY OBJECTIVE

Synergex (EPA Registration No. 1677-250) will be tested to determine efficacy against the food contact surface associated biofilms of *Pseudomonas aeruginosa* ATCC 15442 and *Listeria monocytogenes* ATCC 49594 with the test parameters outlined below. EPA approved modifications to U.S. EPA Microbiology Laboratory Branch (MLB) standard operating procedures MB-19; *Growing a Biofilm using the CDC Biofilm Reactor* and MB-20; *Single Tube Method for Determining the Efficacy of Disinfectants against Bacterial Biofilm* will be the test methods utilized in making the food contact surface biofilm efficacy claim.

Test Parameters

Ecolab SOP number:	MS130; <i>Determination of Antimicrobial Product Efficacy Against Food Contact Surface Associated Biofilm Grown in the CDC Biofilm Reactor Using the Single Tube Test Method</i>
Test Systems:	<i>Pseudomonas aeruginosa</i> ATCC 15442 <i>Listeria monocytogenes</i> ATCC 49594
Carrier Type:	304 stainless steel
Number of Carriers:	5 carriers per organism per batch
Exposure Time:	5 minutes
Exposure Temperature:	Room temperature (21±2°C)
Test Substance Batches:	NB14981-47, NB14981-48, NB14981-49
Test Substance Diluent:	500 ppm Synthetic Hard Water
Test Substance Concentration:	The test substance will be diluted at 1.54 oz/3 gallons to result in the active ingredients at or below the lower limits of 469 ppm hydrogen peroxide, 98 ppm peroxyacetic acid and 24 ppm peroxyoctanoic acid

TEST SUBSTANCE IDENTIFICATION

Test Substance Name: Synergex

Batch Identification: NB14981-47
NB14981-48
NB14981-49

Formula Code: 916834

Date of Manufacture:

Synergex Batch Number	Date of Manufacture
NB14981-47	May 15, 2019
NB14981-48	May 15, 2019
NB14981-49	May 15, 2019

An aliquot of the test substance batches will be retained in the GLP sample storage room at the Ecolab Schuman Campus in Eagan, MN until the quality of the formula no longer affords evaluation. Test substance not dispersed for retention, chemical quality verification or efficacy testing will be stored in Ecolab Microbiological Services cabinet until disposed.

QUALITY ASSURANCE UNIT MONITORING

The protocol, pesticide efficacy in-life and final report are proposed to be inspected by the Ecolab Quality Assurance Unit (QAU) in accordance with their current standard operating procedures. The following proposed Ecolab QA inspections are for planning purposes only and may change. Ecolab QA inspections that are performed, along with their dates and auditors, will be included in the study final report. Changes in Ecolab QA inspections from those proposed below will not require revision of this protocol.

Proposed QAU Monitoring

Protocol Audit
Pesticide Efficacy In-Life Inspection
Final Report Audit

CHEMICAL QUALITY VERIFICATION

Proposed Experimental Start/Termination Dates

Experimental Start Date: August 2019

Experimental Termination Date: August 2019

Method

The chemical quality verification of the test substance concentrates and test substance use-solution were performed under Ecolab GLP study number 1900071. Chemical analysis was performed on each batch of test substance to determine the concentration of the active ingredients. Chemical analysis was also performed on a single batch of test substance use-solution using batch NB14981-47 diluted in sterile lab purified water at 1.54 oz/3 gallons resulting in a use-solution at or below the lower limits of 469 ppm hydrogen peroxide, 98 ppm peroxyacetic acid and 24 ppm peroxyoctanoic acid.

The chemical quality verification was performed by the Analytical Lab using the methods listed below. The methods have been deemed acceptable by the Analytical Lab and the study sponsor to ensure proper characterization of the test substance concentrate and the test substance use-solution. Statistical treatment of test results may be inherent to the method. Additional volumes and dilutions may be necessary to determine the chemistry of the use-solution sample.

QATM 202B: Hydrogen Peroxide and Peracid Analysis by Titration with Potassium Permanganate

Hydrogen peroxide content is determined by an oxidation-reduction titration with potassium permanganate. After the endpoint of this titration has been reached, an excess of potassium iodide is added to the solution. The potassium iodide reacts with peracids to liberate iodine which is titrated with a standard solution of sodium thiosulfate.

QATM 317: Suppressed Peroxide Titration for Peracids and Hydrogen Peroxide

The method requires two steps for the determination of peracids and hydrogen peroxide. The first step determines the peracid content by iodometric titration while suppressing the hydrogen peroxide oxidative property by dilution and cold temperatures (ice water). The presence of ice in the reaction flask does not interfere with the titration chemistry. This method does not distinguish between various types of peracids; it measures the total content of all peracids present.

The second step uses the same sample and measures hydrogen peroxide content by the addition of sulfuric acid and molybdenum catalyst. These two reagents rapidly accelerate the oxidation of iodide by hydrogen peroxide. The hydrogen peroxide concentration is determined by subtracting the difference between the volume of titrant used for the peracid endpoint and the volume required to reach the hydrogen peroxide endpoint.

QATM 337: Peroxyacetic Acid and Peroxyoctanoic Acid Determination by Thiosulfate Titration

The method requires two steps for the determination of peroxyacetic acid and peroxyoctanoic acid. The first step determines the POAA content by filtering out POOA and persulfonated oleic acid (PSOA, if present) while suppressing the hydrogen peroxide by cold temperatures. The presence of deionized ice in the reaction flask does not interfere with the titration chemistry.

The second step rinses the POOA and PSOA (if present) off of the filter with solvent. The PSOA is precipitated using calcium acetate and filtered out of the solution. The POOA can then be measured.

QATM 202B was used to determine the hydrogen peroxide concentration in both the concentrates and use-solution. QATM 317 was used to determine the total peracid concentration in the use-solution. QATM 337 was used to determine the peroxyacetic acid and peroxyoctanoic acid concentrations in the concentrates.

The most current QATMs and product specific Bill of Quality will be used during the course of this study for the chemical analysis.

Interpretation of Results

The concentration of the active ingredients in the test substance concentrates will be judged acceptable for pesticide efficacy testing when within the range specified by the Confidential Statement of Formula (CSF) upper and lower certified limits as seen in the table below.

Test Substance Acceptance Limits		
Active Ingredient	CSF Lower Certified Limit	CSF Upper Certified Limit
Hydrogen Peroxide	9.75%	11.55%
Peroxyacetic Acid	2.04%	2.72%
Peroxyoctanoic Acid	0.49%	0.78%

The peroxyacetic acid and peroxyoctanoic acid active ingredients could not be distinguished nor analyzed at use solution levels using the validated quality assurance test methods. Instead, the total peracid concentration along with hydrogen peroxide were measured in the use solution to ensure the use solution was prepared at or below the lower limits. Per the sponsor and Primary Validation Report of QATM 317 (Document No.: REPT 317-007-022-P), the lower certified limit of total peracid is 2.43%. After applying the 0.401% (1.54 oz/3 gal) dilution for the use solution, the total peracid lower limit concentration in the use solution is 0.0117%.

The concentration of the active ingredients in the test substance use-solution at the lower limit is <1.0%. Therefore, the lower acceptance limit for these active ingredients will be expanded by 10%. The expanded range is based on 40 CFR § 158.350 (Certified Limits) and was calculated as shown below.

$$\begin{aligned} &\text{Calculated Lower Acceptance Limit for Hydrogen Peroxide} \\ &= [0.0469\% - (0.0469 \times 0.1)] = 0.04221\% \end{aligned}$$

$$\begin{aligned} &\text{Calculated Lower Acceptance Limit for Total Peracid} \\ &= [0.0117\% - (0.0117 \times 0.1)] = 0.01053\% \end{aligned}$$

The calculated upper acceptance limit for the active ingredients in the test substance use-solution were determined by adjusting 2% above the lower limit per U.S. EPA Office of Chemical Safety and Pollution Prevention Product Performance Test Guidelines 810.2000: General Considerations for Testing Public Health Antimicrobial Pesticides – Guidance for Efficacy Testing (February 2018) as shown below.

$$\begin{aligned} &\text{Calculated Upper Acceptance Limit for Hydrogen Peroxide} \\ &= [0.0469\% + (0.0469\% \times 0.02)] = 0.04784\% \end{aligned}$$

$$\begin{aligned} &\text{Calculated Upper Acceptance Limit for Total Peracid} \\ &= [0.0117\% + (0.0117\% \times 0.02)] = 0.01193\% \end{aligned}$$

The concentration of the active ingredients in the test substance use-solution will be judged acceptable for pesticide efficacy testing if within the expanded acceptance limits listed in the table below.

Test Substance Use-Solution Acceptance Limits	
Active Ingredient	Expanded Acceptance Limits
Hydrogen Peroxide	0.04221-0.04784%
Total Peracid	0.01053-0.01193%

Note: For test substances with multiple active ingredients, the test substance is diluted so that all active ingredients are at or below the lower limit. As a result of diluting to ensure all active ingredients present are equal to or less than the lower limit, it is possible that some active ingredients may fall below the lower end of the range. It is acceptable if this occurs.

The Chemical Quality Verification results will be reported in the final report of this study.

PESTICIDE EFFICACY TESTING

Proposed Experimental Start/Termination Dates

Experimental Start Date August 2019

Experimental Termination Date October 2019

Methods

Pesticide efficacy data will be generated by the Microbiology Lab using the most current methods listed below. See the specific methods in the Protocol Appendix.

Method Number	Method Name
MS008	<i>Synthetic Hard Water Preparation & Standardization</i>
MS130	<i>Determination of Antimicrobial Product Efficacy Against Food Contact Surface Associated Biofilm Grown in the CDC Biofilm Reactor Using the Single Tube Test Method</i>

Test Method Requirement and Test System Justification

The following apply when determining the effectiveness of an antimicrobial product for biofilm efficacy claims on food contact surfaces; 5 test carriers are required on each of three samples, representing different batches, which are to be evaluated on independent test days. The required organisms are *Pseudomonas aeruginosa* (ATCC 15442) and *Listeria monocytogenes* (ATCC 49594). An adaptation of the U.S. EPA MLB SOPs MB-19 and MB-20 for the above stated organisms will be used to determine biofilm efficacy of an antimicrobial product on food contact surfaces. U.S. EPA Office of Chemical Safety and Pollution Prevention Product Performance Guidelines 810.2200 – Disinfectants for Use on Environmental Surfaces – Guidance for Efficacy Testing (February 2018) also applies to this study.

Test Method Justification

Ecolab Microbiological Services SOP MS130; *Determination of Antimicrobial Product Efficacy Against Food Contact Surface Associated Biofilm Grown in the CDC Biofilm Reactor Using the Single Tube Test Method* will be the test method utilized in this study.

Test Systems and Identification

The test systems which will be utilized for this procedure are *Pseudomonas aeruginosa* ATCC 15442 and *Listeria monocytogenes* ATCC 49594. Identifications will be performed by observing the colony morphology and performing Gram stains.

Organic Soil

No Soil

Statement of Proposed Statistical Method

None

Test Substance Diluent

500 ppm synthetic hard water prepared as described in Ecolab Microbiological Services SOP MS008; *Synthetic hard Water Preparation & Standardization* will be the diluent.

Test Substance Concentration

Antimicrobial efficacy testing will be performed at or below the lower limits of 469 ppm hydrogen peroxide, 98 ppm peroxyacetic acid and 24 ppm peroxyoctanoic acid when diluted at 1.54 oz/3 gallons.

Active Ingredient	CSF Lower Certified Limit	Specific Gravity	Percent Dilution*	Resulting ppm of Active Ingredient
Hydrogen Peroxide	9.75%	1.20	0.401%	469 ppm
Peroxyacetic Acid	2.04%			98 ppm
Peroxyoctanoic Acid	0.49%			24 ppm

*Study proposed dilution: (1.54 oz/3 gallons) x (1 gallon/128 oz) x (100%) = 0.401%

$$\text{Resulting ppm of active ingredient} = \left(\frac{\% \text{Active}}{100\%} \right) \left(\frac{\% \text{Dilution}}{100\%} \right) \times (\text{Specific Gravity}) (10^6)$$

The following calculations will be used to ensure that all active ingredients are at or below their lower limits in the test substance use-solution for use in efficacy testing:

$$\text{Target mass (g) of product} = \frac{(\text{ppm Active at LCL})(\text{Total mass of use-solution})(100\%)}{(10^6)(\% \text{Active Ingredient Result})}$$

Dilution based on % hydrogen peroxide result from QATM 202B:
g of test substance batch in 500 g to yield 469 ppm hydrogen peroxide (H₂O₂) =

$$\frac{(469 \text{ ppm})(500 \text{ g})(100\%)}{(10^6)(\% \text{H}_2\text{O}_2 \text{ in batch})}$$

Dilution based on % peroxyacetic acid result from QATM 337:
g of test substance batch in 500 g to yield 98 ppm peroxyacetic acid (POAA)=

$(98 \text{ ppm})(500 \text{ g})(100\%)$
 $(10^6) (\% \text{POAA in batch})$

Dilution based on % peroxyoctanoic acid result from QATM 337:
g of test substance batch in 500 g to yield 24 ppm peroxyoctanoic acid (POOA)=

$(24 \text{ ppm})(500 \text{ g})(100\%)$
 $(10^6) (\% \text{POOA in batch})$

Dilution procedure for efficacy testing (or equivalent dilution)

Synerex Batch Number	Active Ingredient Concentration ¹	Test Substance Weight*: Diluent Weight*
NB14981-47	Hydrogen Peroxide: 10.80%	2.17 g : 497.83 g
	Peroxyacetic Acid: 2.37%	2.07 g : 497.93 g
	Peroxyoctanoic Acid: 0.57%	2.11 g : 497.89 g
NB14981-48	Hydrogen Peroxide: 10.78%	2.18 g : 497.82 g
	Peroxyacetic Acid: 2.32%	2.11 g : 497.89 g
	Peroxyoctanoic Acid: 0.57%	2.11 g : 497.89 g
NB14981-49	Hydrogen Peroxide: 10.89%	2.15 g : 497.85 g
	Peroxyacetic Acid: 2.34%	2.09 g : 497.91 g
	Peroxyoctanoic Acid: 0.54%	2.22 g : 497.78 g

*Weights may vary by $\pm 0.03 \text{ g}$

The dilution in **bold** will be used for efficacy testing

¹ Active Ingredient Concentration determined under Ecolab GLP Study number 1900071

Test Surface

304 stainless steel coupons (1.27 \pm 0.013 cm diameter, approximate 3.0 mm thickness)

Exposure Time/Temperature

The test systems will be exposed to the test substance for 5 minutes at room temperature (21 \pm 2°C)

Neutralizer Medium

36 mL D/E Neutralizing Broth

Plating Medium

R2A (*Pseudomonas aeruginosa* ATCC 15442)
BHI (*Listeria monocytogenes* ATCC 49594)

Incubation Time/Temperature

Test plates will be incubated for 72±4 hours at 35±2°C. Control plates will be incubated for 48±4 hours at 35±2°C.

Test Controls

The following controls will be incorporated with the test procedure for each test system:

- a. Control Carrier Enumeration
- b. Test Substance Diluent Sterility
- c. Neutralization Confirmation
- d. Test System Purity
- e. Continuous Flow Operation Media Sterility Control

Details on each of the above controls can be found in Ecolab SOP MS130 located in the Protocol Appendix.

Interpretation of Test Results

The performance standard for evaluation of an antimicrobial pesticidal product against biofilm associated with food contact surfaces is ≥6 log reduction in viable *Pseudomonas aeruginosa* biofilm and ≥5 log reduction in viable *Listeria monocytogenes* biofilm from test carriers as compared to the control carriers.

DATA RETENTION

Following the completion of the study, the original final report and raw data will be archived at the Ecolab Schuman Campus in Eagan, Minnesota or at an approved off-site location. All records that would be required to reconstruct the study and demonstrate adherence to the protocol will be maintained for the life of the commercial product plus four years.

TEST SUBSTANCE RETENTION

An aliquot of each batch of test substance will be retained in the GLP sample storage room at the Ecolab Schuman Campus in Eagan, Minnesota until the quality of the formula no longer affords evaluation.

GOOD LABORATORY PRACTICES

This study will be conducted according to Good Laboratory Practices, as stated in 40 CFR Part 160. If it becomes necessary to make changes in the approved protocol, the revisions and reasons for change will be documented, reported to the sponsor and will become part of the permanent file for that study. The sponsor will be notified as soon as it is practical whenever an event occurs that could have an effect on the validity of the study.

- **Name and Address of Sponsor**


Christina Ramsumair
Ecolab Schuman Campus
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Eagan, MN 55121

- **Name and Address of Performing Laboratory**

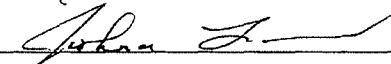
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- **Name and Address of Study Director**

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Sponsor

8-27-2019
Date


Study Director

8-27-19
Date

PROTOCOL APPENDIX

Microbiological Services (MS) Methods:

MS008	<i>Synthetic Hard Water Preparation & Standardization</i>
MS130	<i>Determination of Antimicrobial Product Efficacy Against Food Contact Surface Associated Biofilm Grown in the CDC Biofilm Reactor Using the Single Tube Test Method</i>
MS102	Attachment

ECOLAB – MICROBIOLOGICAL SERVICES

TITLE: Synthetic Hard Water Preparation & Standardization

LEGACY: MS008

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1.0 PURPOSE

To describe how to prepare standardized synthetic hard water solution to be used for diluting products that possess hard water claims.

2.0 SYNTHETIC HARD WATER PREPARATION

2.1 Fill out a media preparation sheet for Solution A and Solution B. Retain in the Media Preparation Log Book. Prepare 1 L of each solution or alternate amount with proportional ingredients.

2.2 Solution A Preparation

Magnesium Chloride ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$)	67.74 \pm .1 g
Calcium Chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$)	97.99 \pm .1 g
Sterile Lab Purified Water	1 L

2.2.1 Dissolve powders in 600 mL of boiled lab purified water, and then bring to 1 L volume in a 1 L volumetric flask after solution has cooled.

2.2.2 Dispense into appropriate containers (for example, 250 mL Pyrex screw cap bottles) and autoclave for ≥ 15 minutes at $\geq 121^\circ\text{C}$.

2.2.3 Label using the standard Ecolab labels with a 1 month expiration date and store at $2 - 8^\circ\text{C}$.

2.2.4 Quality Control

2.2.4.1 Visual: Clear solution

2.2.4.2 Sterility Check: Sterile after incubation at $32 \pm 2^\circ\text{C}$ for ≥ 5 days

2.2.4.3 Expiration Date: One month at $2 - 8^\circ\text{C}$

2.3 Solution B Preparation

Sodium Bicarbonate (NaHCO_3)	56.03 \pm .1 g
Sterile Lab Purified Water	1 L

2.3.1 Dissolve in 600 mL of boiled lab purified water, then bring to 1 L volume in a 1 L volumetric flask with lab purified water after solution has cooled.

2.3.2 Filter sterilize through a 0.45 micron filter into appropriate sterile containers. (approximately 150 – 200 mL per container)

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- 2.3.3 Label using the standard Ecolab labels with a one month expiration date and store at 2 – 8°C.
- 2.3.4 Quality Control
 - 2.3.4.1 Visual: Clear solution
 - 2.3.4.2 Sterility Check: Sterile after incubation at $32 \pm 2^{\circ}\text{C}$ for ≥ 5 days
 - 2.3.4.3 Expiration Date: One month at 2 – 8°C
- 2.4 Hard Water Preparation
 - 2.4.1 To avoid precipitation of the hard water solution, water should be at room temperature before the addition of Solutions A or Solution B.

Total hardness as ppm $\text{CaCO}_3 = 2.495 \times \text{ppm Ca} + 4.115 \times \text{ppm Mg}$
 - 2.4.2 To each 1 L of water to be prepared add 1 mL of Solution A for each 100 ppm of CaCO_3 hardness desired plus 4 mL of Solution B (e.g. for 500 ppm synthetic hard water add 5 mL of Solution A and 4 mL of Solution B per liter of water).
 - 2.4.3 Bring to 1 L volume with sterile lab purified water. If preparing more than 1 L, combine flasks in a sterile 4 L beaker blender after adding appropriate amounts of Solutions A and Solution B and bringing to volume.
- 2.5 Alternate Hard Water Preparation: Commercial Preparation
 - 2.5.1 Use a commercially available standard, preferably NIST traceable, to prepare synthetic hard water (e.g. Hach Chemical Company 218710).
 - 2.5.2 To prepare a 400 ppm as CaCO_3 solution, add four ampules of 10,000 ppm as CaCO_3 standard (10 mL each ampule) to a 1 L volumetric flask.
 - 2.5.3 Add sterile lab purified water up to 1 L mark. Solutions of other water hardness and different volumes may be prepared as appropriate.
- 2.6 The pH of all test waters less than 2000 ppm hardness (as CaCO_3) should be 7.6 – 8.0. Adjustment of hard water pH using NaOH or HCl may be necessary depending on the starting water pH.

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3.0 STANDARDIZATION OF SYNTHETIC HARD WATER

3.1 Method Check – Prior to standardization of the synthetic hard water, the accuracy of the titration method must be checked by analyzing a 500 ppm CaCO_3 standard. This must be performed on a monthly basis or when testing new batches of Solution A and Solution B.

3.1.1 Dilute 10 mL of a 1000 ppm CaCO_3 standard (1 mL = 1 mg CaCO_3) in 10 mL of lab purified water to result in a 500 ppm CaCO_3 solution.

3.1.2 Dilute 10 mL of the 500 ppm CaCO_3 solution in 40 mL of lab purified water in a beaker.

3.1.3 Test solution as described in 3.2.2 – 3.2.5.

3.1.4 The hardness of the 500 ppm solution is determined as follows:

$$\text{hardness (ppm)} = (\text{mL EDTA}) \times 100$$

3.1.5 Record the result and the lot number of the standard on Form 3011. Hardness of the 500 ppm CaCO_3 solution must be 500 ± 20 ppm CaCO_3 . Failure of the standard to fall within this range indicates a problem in the test method. Corrective actions should be documented in the comments section on Form 3011. The procedure may be used for standardization of synthetic hard water only when results of the standard are within the range described above.

3.1.6 Records from the current and previous year will be kept in the Microbiological Services Equipment Maintenance binder. All earlier records will be archived in the first quarter of the current year. For example, records from 2016 will be archived by March of 2018. Records will be transferred to Ecolab Archives at the Ecolab Schuman Campus in Eagan, MN or to an approved off-site location.

3.2 Sample Testing/Standardization

3.2.1 Dilute 10 mL of prepared hard water in 40 mL of lab purified water in a beaker.

3.2.2 Add 1 mL water hardness buffer with magnesium. Use hood when adding; the buffer has irritating vapors.

3.2.2.1 The buffer is VWR product code VW3491 (or equivalent)

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3.2.2.2 Approximate composition of buffer, % by weight:

Ammonia	56-57
Ammonium chloride	6-7
EDTA-Magnesium Tetraacetate Salt	0.5
Water	> 35

Note: This buffer has a relatively short expiration.

- 3.2.3** Optional: Add 1 mL inhibitor – needed only if previous titration without it has been unsatisfactory (refer to 3.2.5.2).
- 3.2.4** Add just enough Ecolab hardness indicator #016 to yield a pink coloration upon dissolving.
- 3.2.4.1** Hardness indicator 016 contains Calgamite (1-(1- hydroxy-4-methyl-2-phenylazo)-2-naphthol-4-sulfonic acid) as the actual indicator, along with inert ingredients.
- 3.2.4.2** It is obtained from Ecolab Test Kits (order through F&B Customer Service) at the Ecolab Engineering Center.
- 3.2.5** Add 0.01M EDTA slowly until the pink coloration turns blue. Record the number of milliliters of EDTA needed to create the color change.
- 3.2.5.1** The titration should be completed within five minutes of buffer addition to minimize tendency toward CaCO_3 precipitation.
- 3.2.5.2** If the end point color change is not clear and sharp (e.g. the color changes to blue and then drifts back to pink) then an inhibitor/complexing agent must be added (or possibly, the indicator has deteriorated).
- 3.2.5.3** Prepare inhibitor solution by dissolving 5.0 g sodium sulfide nonahydrate ($\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$) or 3.7 g $\text{Na}_2\text{S} \cdot 5\text{H}_2\text{O}$ in 100 mL distilled water. Prepare and dispense in hood. This inhibitor solution deteriorates quickly though air oxidation and should be made each day it is needed.
- 3.2.5.4** Dilute new sample of test solution and re-titrate beginning with step 3.2.2, including addition of inhibitor.

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TITLE: Synthetic Hard Water Preparation & Standardization

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3.2.6 The hardness of the water is determined as follows:

$$\begin{aligned}\text{Hardness as mg CaCO}_3/\text{L} &= (\text{mL EDTA} \times 1000)/10 \text{ mL of sample} \\ &= \text{mL EDTA} \times 100\end{aligned}$$

3.2.7 Upon titration, hardness must not exceed 20 ppm above or below the ppm specified in test procedure/protocol/lab statement. Therefore, if a claim is for 500 ppm, the titration must yield 500 ± 20 ppm. If ppm hardness is out of the established range, the sample should be retitrated. Upon a second titration, if ppm hardness is still outside established ranges, the hard water must be diluted or additional solution added to yield the desired ppm. After adjustments have been made, the water must be titrated to determine ppm hardness.

3.2.8 Only two adjustments may be made to the hard water following the above procedure. If the hard water is outside the established limits after two adjustments, the water must be disposed of and the process reinitiated.

3.2.7 For GLP testing, record Hard Water Preparation and Standardization on Form 3010 or Form 3113.

4.0 RELATED FORMS

- 4.1 Form 3010: Synthetic Hard Water Preparation & Standardization
- 4.2 Form 3011: Water Hardness Standard Results
- 4.3 Form 3072: Solution A Prep Log
- 4.4 Form 3074: Solution B Prep Log
- 4.5 Form 3113: Test Substance Use-Solution Preparation for Analysis

5.0 REFERENCES

- 5.1 AOAC (2011) Method 960.09 (E)
- 5.2 APHA, Standard Methods for the Examination of Water & Wastewater, 21st Ed., 2005. Section 3500-Ca B. EDTA Titrimetric Method.

6.0 MOST RECENT REVISION SUMMARY

In 2.1, added option to prepare amount other than 1 L of Solution A or Solution B.

ECOLAB – MICROBIOLOGICAL SERVICES

TITLE: Determination of Antimicrobial Product Efficacy Against Food Contact Surface Associated Biofilm Grown in the CDC Biofilm Reactor Using the Single Tube Test Method

LEGACY: MS130

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1.0 PURPOSE

To evaluate the efficacy of antimicrobial products intended for use on food-contact surfaces using the single tube test method against biofilm grown in the CDC Biofilm Reactor.

2.0 BIOFILM REACTOR APPARATUS

2.1 CDC Biofilm Reactor Components

- 2.1.1 Berzelius borosilicate glass tall beaker – 1000 mL without pour spout, 9.5 ± 0.5 cm diameter. Barbed effluent spout added at 400 ± 50 mL mark. Spout angled 30° to 45° to ensure sufficient drainage. Spout should accommodate flexible tubing with an inner diameter (ID) of 8 – 11 mm.
- 2.1.2 Reactor top – Ultra-high molecular weight (UHMW) polyethylene top (10.1 cm diameter tapering to 8.33 cm) equipped with a minimum of three holes accommodating 6 – 8 cm long pieces of stainless steel or other rigid autoclavable tubing with an outer diameter (OD) of 5 – 8 mm for media inlet, air exchange and inoculation port. Center hole, 1.27 cm diameter, to accommodate the glass rod used to support the baffle assembly. Eight rod holes, 1.905 cm diameter, notched to accommodate stainless steel rod alignment pin (0.236 cm OD).
- 2.1.3 Polypropylene rods – Eight polypropylene rods, 21.08 cm long, machined to hold three coupons at the immersed end. Three 316 stainless steel set screws imbedded in the side to hold coupons in place. Rods to fit into holes in reactor top and lock into preformed notches with alignment pin. When 24 coupons are not needed, blank polypropylene rods may also be used. Blank rods are machined without coupon recesses and can be used as a rod place holder.
- 2.1.4 Coupons – Up to twenty-four cylindrical 304 stainless steel coupons with a diameter of 1.27 ± 0.013 cm and thickness of approximately 3.0 mm. Alternate coupon materials may be substituted where appropriate. Coupons may be purchased from BioSurface Technologies, Corp. (www.biofilms.biz) or made by the User to the appropriate size specifications listed.

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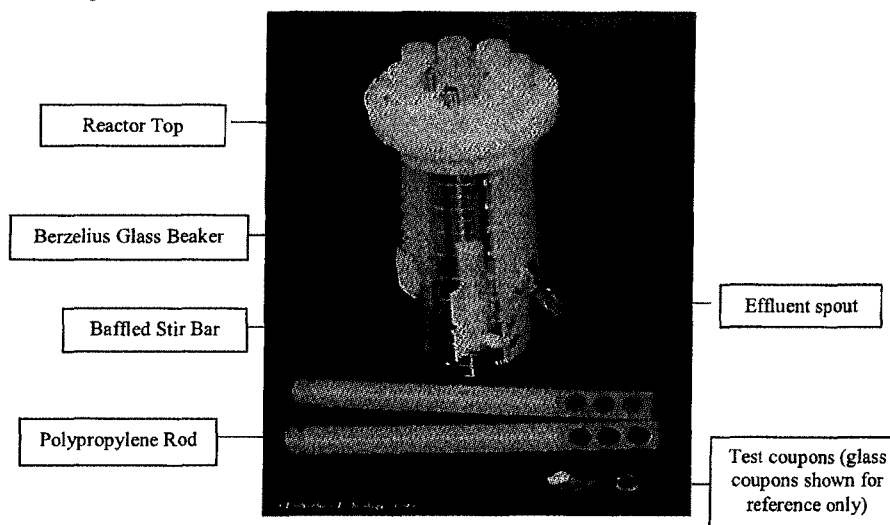
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- 2.1.5 Stir blade assembly (Baffled Stir Bar) – Teflon blade (5.61 cm) fitted into cylindrical Teflon holder (8.13 cm) and held in place with a magnetic stir bar (2.54 cm). Teflon holder fits onto a glass rod (15.8 cm), fitted into the reactor top. The glass rod is held in place with a compression fitting and acts as a support for the moving blade assembly.

Note: At this time, the sole source of the CDC Biofilm Reactor is BioSurface Technologies, Corp. (www.biofilms.biz).

- 2.1.6 Small Allen Wrench – For tightening and loosening reactor rod set screws [i.e. 0.050" (1.27 mm) diameter hex head].
- 2.1.7 See Figure 1 for completed CDC Biofilm Reactor assembly.

Figure 1



- 2.2 Glass flow break – any that will connect with tubing of ID 3.1 mm and withstand sterilization.
- 2.3 Silicone tubing – two sizes of tubing; must withstand sterilization. Tubing may be used multiple times, however, should be replaced as necessary when signs of wear are noticed.

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- 2.3.1 One with ID of 3.1 mm and OD of 3.2 mm [used for most connections between continuous flow (CSTR) growth medium carboy and the reactor]. Example: Masterflex L/S 16 size tubing.
- 2.3.2 One with ID of 7.9 mm and OD of 9.5 mm (used for reactor effluent from port to waste carboy). Example: Masterflex L/S 18 size tubing.
- 2.4 Norprene Tubing – with ID of 3.1 mm and OD of 3.2 mm. For use in the peristaltic pump. Example: Masterflex Norprene tubing, L/S 16 size tubing.
- 2.5 Plastic tubing adaptors (*if necessary*) – for splicing in flow break and nutrient tubing line to biofilm reactor. Size should be appropriate for diameter of tubing.
- 2.6 Carboys – Two 20 liter autoclavable carboys, one used for reactor waste and one for sterile growth medium.
 - 2.6.1 Carboy lids – Two carboy lids, each with at least two barbed fittings to accommodate tubing of at least a 3.1 mm ID. One lid will be for sterile growth medium carboy with one barb to be used for bacterial air vent and one barb for growth medium line. One lid will be for waste carboy with one barb to be used for reactor waste and one barb for bacterial air vent. Carboy lids may be purchased with appropriate barbed fittings (i.e. Versatile Cap).
- 2.7 Bacterial Air Vent (Filter) – Sterile 0.2 µm pore size filter, to be spliced into tubing on waste carboy, growth medium carboy and reactor top (recommended diameter of 37 mm).
- 2.8 Magnetic Stir Plate – With an appropriate size top plate and capable of rotating at a range of 60 – 125 ± 5 RPM.
- 2.9 Peristaltic Pump – With pump head that can hold tubing with ID of 3.1 mm and OD of 3.2 mm.
- 2.10 Clamp – Extension clamp, used to hold flow break vertically.
- 2.11 Clamp Stand – Of appropriate height to be used with extension clamp to suspend glass flow break vertically and stabilize tubing above reactor during operation.
- 2.12 Tubing Clamps – For clamping reactor effluent tube during Batch Phase growth.

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3.0 CULTURE MEDIA (propagation)

3.1 Brain Heart Infusion (BHI) Broth– various concentrations of BHI are used for *L. monocytogenes* inoculum preparation, Batch Phase and Continuous Flow (CSTR) growth.

3.1.1 37 g/L (Stock) BHI – for *L. monocytogenes* inoculum preparation and use in preparation of CSTR media.

3.1.2 3.7 g/L (10%) BHI – for *L. monocytogenes* Batch Phase reactor operation.

3.1.3 1.85 g/L (5%) BHI – for *L. monocytogenes* Continuous Flow reactor operation.

3.2 Tryptic Soy Broth (TSB) – various concentrations of TSB are used for *P. aeruginosa* inoculum preparation, Batch Phase and Continuous Flow (CSTR) growth.

3.2.1 40 g/L TSB – concentrated TSB for use in preparation of CSTR media.

3.2.2 30 g/L (Stock) TSB – for use in *P. aeruginosa* inoculum preparation (Neutralization Confirmation Control).

3.2.3 300 mg/L TSB – for *P. aeruginosa* inoculum preparation and Batch Phase reactor operation.

3.2.4 100 mg/L TSB – for *P. aeruginosa* Continuous Flow reactor operation.

3.3 Other media suitable for culturing alternate test organisms.

4.0 SUBCULTURE MEDIA (plating)

4.1 Brain Heart Infusion (BHI) agar – for *L. monocytogenes*

4.2 R2A agar - for *P. aeruginosa*

4.3 Other media suitable for culturing alternate test organisms.

5.0 NEUTRALIZER

5.1 D/E Neutralizing broth

5.2 Other appropriate neutralizer

6.0 REAGENT & APPARATUS

6.1 Phosphate buffered dilution water (PBDW)

6.2 Ethanol – used to flame-sterilize Allen wrench

6.3 Bunsen burner

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- 6.4 Pipettes/transfer device
- 6.5 Sterile disposable pipettes
- 6.6 Micropipettor with sterile disposable tips
- 6.7 Sterile plate spreaders
- 6.8 Balance
- 6.9 Ultrasonic Water Bath – any cavitating sonicating bath that operates at 45 ± 5 kHz
- 6.10 Vortex mixer
- 6.11 Inoculating loops
- 6.12 Sterile forceps
- 6.13 Sterile test tubes
- 6.14 Test tube racks
- 6.15 Conical tube racks
- 6.16 Sterile conical tubes – 50 mL or 250 mL polypropylene sterile screw cap centrifuge tubes.
- 6.17 Sterile conical tube Splashguards

Note: Splashguard inserts may be purchased from BioSurface Technologies or built by the User to the appropriate specifications which ensure the bottom of the Splashguard sits at the straight/conical interface of the treatment tube. Material of construction must be autoclavable.

- 6.18 Water Bath
- 6.19 Incubator
- 6.20 Appropriate glassware/plasticware
- 6.21 Polyethersulfone membrane filter (PES) – 47 mm diameter and 0.45 μ m pore size (i.e. Pall 100 mL Supor® White, 0.45 μ m filters). For recovery of test organism from treated coupons. Filtration units (reusable or disposable) may be used.
- 6.22 Timer
- 6.23 Biosafety cabinet
- 6.24 Laboratory detergent (e.g. Micro-90 Concentrated Cleaning Solution or equivalent lab detergent)
- 6.25 24 well flat bottom culture plates (if necessary) – for cleaning of coupons.

7.0 DEFINITIONS

- 7.1 Batch phase – Establishment of the biofilm by operating the reactor without the flow of nutrients (growth medium), but with constant shear force caused by the rotating baffle.

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- 7.2 Continuously stirred tank reactor (CSTR) or Continuous Flow phase – Establishment of a steady state biofilm population achieved with the continuous flow of nutrients (growth medium) into the reactor and continual removal of waste.
- 7.3 Biofilm – Microorganisms living in a self-organized community attached to surfaces, interfaces, or each other, embedded in a matrix of extracellular polymeric substances of microbial origin, while exhibiting altered phenotypes with respect to growth rate and gene transcription.

8.0 PREPARATION OF REACTOR & COUPONS

8.1 CDC Biofilm Reactor calibration and standardization.

8.1.1 Determine the operating volume of reactor prior to use in testing.

- 8.1.1.1 For new reactors not listed in MS102 Attachment, fully assemble the reactor (including rods with coupons and baffle) and place on a stir plate set to the appropriate speed. Clamp tubing on the reactor effluent port.
- 8.1.1.2 Remove one of the rods and fill the reactor with a known volume of water, higher than the level of the glass effluent spout (i.e. 500 mL) and reinsert the rod. Turn on the stir plate. Perform at 125 ± 5 and/or 60 ± 5 RPM, as necessary.
- 8.1.1.3 Remove effluent tubing clamp and collect the excess fluid that drains out of the reactor.
- 8.1.1.4 Measure the approximate volume of water that drains; the remaining water in the reactor is the operating volume (Operating Volume = Starting volume of water – Drained volume of water).
- 8.1.1.5 Refer to the MS102 Attachment for reactor operating volumes determined prior to use in testing.

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8.1.2 Determination of required pump flow rate.

8.1.2.1 For *L. monocytogenes*, use the operating volume determined in 8.1.1 and a residence time of 60 minutes to calculate the appropriate pump flow rate required for biofilm formation. For *P. aeruginosa*, use the operating volume determined in 8.1.1 and a residence time of 30 minutes to calculate the appropriate pump flow rate required for biofilm formation. Using the formula $Q = V/RT$, where Q = flow rate (volume of liquid which passes through the tubing into the reactor per unit of time), V = operating volume of reactor and RT = residence time (e.g. if the operating volume is 325 mL and the residence time is 30 minutes, then the pump flow rate should be set to equal 10.8 ± 0.2 mL/minute). Include the allowance of ± 0.2 mL/min for calculated pump flow rate.

8.1.2.2 For alternate test organisms, follow the procedure listed in 8.1.2.1; however, an alternate residence time may be followed as needed.

8.2 Preparation of Coupons

8.2.1 Coupons may be used repeatedly with proper cleaning and screening between each use. Following use in the reactor, place contaminated coupons in an appropriate vessel (e.g. small beaker), cover with liquid (i.e. water) and autoclave with the other parts of the contaminated reactor system.

8.2.2 For initial use (new coupons) and re-use, sonicate coupons individually (e.g. in plastic 50 mL conical tubes or 24 well plates) for five minutes in a 1:100 dilution of laboratory detergent and water. The soapy water must completely cover the coupons.

8.2.3 Rinse coupons with deionized water and sonicate for approximately 1 minute.

8.2.4 Repeat rinsing and one minute sonication with deionized water until no soap is left on the coupons. Once coupons are clean, wear gloves when handling the coupons to prevent oils and/or other residues from contaminating the surface.

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8.2.5 Check each coupon for scratching, chipping, other damage or accumulation of debris before use. If applicable, carrier screening may be performed by observing carriers under at least 20X magnification (i.e. use of stereoscope or dissecting microscope). Discard any carriers which demonstrate any visible damage to surface topography. Carrier processing shall be recorded on Form 3170.

8.2.6 Use the screened and cleaned coupons for Preparation of CDC Biofilm Reactor in 8.3, or store in a Petri dish for future use.

8.2.7 Where necessary, additional or alternate coupon preparation procedures may be followed (e.g. for alternate carrier types).

8.3 Preparation of CDC Biofilm Reactor

8.3.1 Invert the reactor top and place baffled stir bar onto the glass rod positioned in the center of the reactor top.

8.3.2 Invert the beaker and place onto the assembled top. Turn the reactor over so that the reactor top is upright.

8.3.3 Place a cleaned and screened coupon into each hole in the reactor rod, leaving the coupon flush with the inside rod surface. Tighten set screw. Determine the appropriate number of carriers that will be needed for testing and prepare the appropriate number of reactor rods.

8.3.4 Place rods loosely into the reactor top (not yet fitted into notches, to allow for pressure to escape during sterilization). If eight coupon-containing rods are not needed for testing, substitute blank rods to ensure a total of eight rods are fitted into the reactor top.

8.3.5 Connect the bacterial air vent by fitting the vent to a small section of appropriately sized tubing (i.e. L/S 18 size tubing) and attach to one of the available rigid tubes on the reactor top.

8.3.6 Attach an appropriate length of tubing (i.e. L/S 18 size tubing) from the reactor effluent port. Ensure length is sufficient to reach waste carboy.

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8.4 Sterilization of the CDC Biofilm Reactor system

- 8.4.1 Cover any reactor top openings and the overflow (waste) tubing with aluminum foil.
- 8.4.2 Sterilize the empty reactor system for 20 minutes in a steam sterilizer.
- 8.4.3 Following sterilization, verify that all coupons remained intact in the rods. If a coupon has fallen out of a rod, the coupon can be replaced, and the reactor may be re-sterilized. Or, the rod(s) and coupon(s) may be aseptically removed from the reactor and replaced with a sterile blank rod(s).

9.0 CULTURE PREPARATION

Note: For *P. aeruginosa*, only frozen stock cultures prepared following the OECD preparation method should be used. For *L. monocytogenes* or any other alternate test organism(s), frozen stock cultures may be prepared as appropriate. See MS032 for reference to frozen stock preparation methods.

- 9.1 For *Pseudomonas aeruginosa* (ATCC 15442), defrost a single cryovial and briefly vortex to mix. Add 10 µL of the thawed frozen stock (single use) to a tube containing 10 mL of TSB (300 mg/L) and vortex to mix. Incubate the bacterial suspension at 35 ± 2°C for 24 ± 2 hours.
- 9.2 For *Listeria monocytogenes* (ATCC 49594), defrost a single cryovial and briefly vortex to mix. Add 10 µL of the thawed frozen stock (single use) to a tube containing 10 mL of BHI (37 g/L – stock BHI) and vortex to mix. Incubate the bacterial suspension at 35 ± 2°C for 24 ± 2 hours.
- 9.3 For alternate test organisms, modification of culture medium/concentration, incubation time and incubation temperature may be necessary.
- 9.4 Following incubation, verify purity of the tube by streaking a loopful of organism suspension onto the appropriate agar for isolation. Incubate plate overnight at 35 ± 2°C and examine for purity of test organism.

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10.0 GENERATION OF BIOFILM IN CDC BIOFILM REACTOR

10.1 Batch Phase Growth

10.1.1 Making sure the overflow (waste line) is clamped, aseptically add 500 mL of the batch culture medium to the cooled reactor by aseptically removing a rod from the reactor, adding the medium through the opening and re-inserting the rod. Ensure all rod alignment pins are set in the reactor top notches. To help minimize contamination, it is recommended to perform this step in a biosafety cabinet.

10.1.1.1 For *P. aeruginosa*, the batch phase medium is **300 mg/L TSB**. Batch phase medium may be prepared at this concentration prior to use or may be prepared on day of inoculation by adding 5 mL of 30 g/L TSB to 495 mL of sterile lab purified water, or equivalent ratio. Alternate concentrations of TSB may be used where appropriate to achieve the final concentration of 300 mg/L TSB.

10.1.1.2 For *L. monocytogenes*, the batch phase medium is **3.7 g/L (10%) BHI**. Batch phase medium may be prepared at this concentration prior to use or may be prepared on day of inoculation by adding 50 mL of stock (37 g/L) BHI to 450 mL of sterile lab purified water, or an equivalent ratio. Alternate concentrations of BHI may be used where appropriate to achieve the final concentration of 3.7 g/L BHI.

10.1.1.3 For alternate organisms, use a batch phase medium and concentration that is appropriate for growth.

10.1.2 Aseptically inoculate the reactor by adding 1 mL of the vortex mixed 24 ± 2 hour culture from section 9.0 into the batch medium.

10.1.3 Place prepared, inoculated reactor onto stir plate and turn it on.

10.1.3.1 For *P. aeruginosa*, the rotational speed of the baffle is 125 ± 5 RPM. Run the reactor system in batch phase at room temperature (e.g. $21 \pm 2^\circ\text{C}$) for 24 ± 2 hours.

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10.1.3.2 For *L. monocytogenes*, the rotational speed of the baffle is 60 ± 5 RPM. Run the reactor system in batch phase at $35 \pm 2^\circ\text{C}$ for 24 ± 2 hours.

10.1.3.3 For alternate organisms, use a baffle speed rotation and incubation temperature/time that is appropriate for growth.

10.1.4 Confirm appropriate baffle speed rotation by manually counting the number of baffle rotations per minute.

10.2 Continuous Flow Operation (CSTR)

10.2.1 Ensure the nutrient tubing line with spliced in flow break is appropriately and aseptically connected to the Continuous Flow medium and reactor. Clamp flow break in a vertical position.

10.2.2 If not yet performed, remove the foil, place the end of the effluent tubing to a waste carboy and allow reactor to drain by unclamping the tubing. See Figure 2 for schematic of completely assembled reactor system.

10.2.3 Pump a continuous flow of growth medium into the reactor to achieve the required residence time based on the reactor's operating volume. Use calculated flow rate as determined in 8.1.2.

10.2.4 *P. aeruginosa* Continuous Flow Operation

10.2.4.1 Add 50 mL of sterile **40 g/L** TSB to approximately 19 L of sterile water (in carboy), then, if necessary, fill to approximately 20 L with additional sterile lab purified water to achieve a final CSTR growth medium concentration of **100 mg/L** TSB. Alternate TSB concentrations and volumes may be used where appropriate, to ensure a final growth medium concentration of 100 mg/L. Thoroughly mix the contents.

10.2.4.2 Run the Continuous Flow Operation biofilm growth phase at room temperature (e.g. $21 \pm 2^\circ\text{C}$) and 125 ± 5 RPM for 24 ± 2 hours.

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10.2.5 *L. monocytogenes* Continuous Flow Operation

10.2.5.1 Add 1L of sterile stock (37 g/L) BHI to approximately 19 L of sterile water (in carboy), then, if necessary, fill to approximately 20 L with additional sterile lab purified water to achieve a final CSTR growth medium concentration of 1.85 g/L (5%) BHI. Alternate BHI concentrations and volumes may be used where appropriate, to ensure a final growth medium concentration of 1.85 g/L BHI. Thoroughly mix the contents.

10.2.5.2 Preheat the 1.85 g/L (5%) BHI to $35 \pm 2^{\circ}\text{C}$ prior to it entering the reactor. It is recommended that the approximate 20 L of 1.85 g/L BHI be prepared 1 – 2 days prior to CSTR operation and allowed to equilibrate prior to beginning CSTR operation.

10.2.5.3 Run the Continuous Flow Operation biofilm growth phase at $35 \pm 2^{\circ}\text{C}$ and 60 ± 5 RPM for 24 ± 2 hours.

*Note: It is recommended to perform biofilm growth of *L. monocytogenes* in an incubator large enough to contain the reactor, pump, stir plate and all necessary carboys. Where this is not possible (i.e. carboys do not fit in incubator), the following method may be followed: (1) place the Continuous Flow growth medium carboy into an incubator at least one day prior to use to bring the medium to $35 \pm 2^{\circ}\text{C}$. (2) coil ~ 12 to 15 feet of nutrient line tubing inside the incubator housing the reactor/stir plate and prime the tubing with medium at least one day prior to use.*

10.2.6 Alternate Organism Continuous Flow Operation

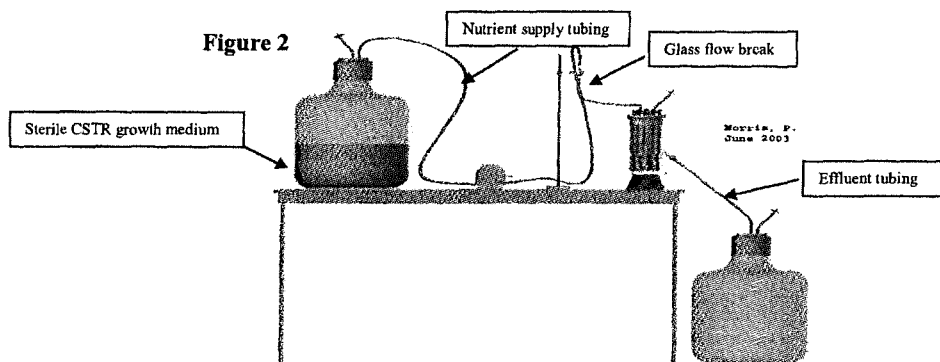
10.2.6.1 Perform Continuous Flow Operation with CSTR medium, concentration, baffle speed, incubation temperature and incubation time as appropriate for organism growth.

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Assembled biofilm reactor system with nutrient and effluent containers

11.0 HARD WATER PREPARATION

- 11.1 If the product possesses a hard water claim, prepare the test substance dilution in synthetic hard water (refer to MS008).

12.0 TEST SUBSTANCE PREPARATION

- 12.1 Prepare test substance use solution ≤ 3 hours prior to use, unless otherwise specified. Prepare test substance dilution in a sterile vessel according to instructions (e.g. if a 1:128 dilution is required, add 1 part test material + 127 parts diluent). If the test substance requires dilution, use ≥ 1.0 mL or ≥ 1.0 g of test material to prepare, unless otherwise directed.

Three batches of product at the Lower Certified Limit (LCL) must be tested against both *P. aeruginosa* (ATCC 15442) and *L. monocytogenes* (ATCC 49594). Each batch of test substance must be evaluated on independent test days; however, each batch may be tested against *P. aeruginosa* and *L. monocytogenes* on the same test date.

- 12.2 If necessary, place prepared test substance in water bath for ≥ 10 minutes to allow product to equilibrate. Evaluation of test substance will be performed at room temperature ($21 \pm 2^\circ\text{C}$) or alternate exposure temperature in a water bath as necessary.

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13.0 EVALUATION OF TEST SUBSTANCE AGAINST BIOFILM

- 13.1 Ensure the neutralizer is at room temperature prior to use.
- 13.2 Turn off the CSTR medium flow and baffled stir bar. Remove coupons from reactor for testing within one hour once growth medium and stir bar have been turned off.
- 13.3 Aseptically remove a randomly selected rod containing coupons with biofilm from the CDC Biofilm Reactor by firmly pulling it straight out of the reactor.
- 13.4 Rinse the coupons to remove planktonic cells.
 - 13.4.1 Orient the rod in a vertical position directly over a 50 mL conical tube containing approximately 30 mL of sterile PBDW.
 - 13.4.2 Immerse the rod with one continuous motion into the PBDW with minimal to no splashing, then immediately remove.
 - 13.4.3 Use a new 50 mL conical tube with approximately 30 mL of sterile PBDW for each rod.
- 13.5 Hold the rod with one of the randomly selected coupons centered over an empty, sterile conical tube (50 mL or 250 mL) containing a splashguard (for coupons exposed to test substance; splashguards are not required for control coupons). Do not allow the rod to contact the tube or splashguard.
- 13.6 Loosen the set screw using a flame-sterilized Allen wrench and allow the coupon to drop directly to the bottom of the tube. If the coupon does not freely drop, press directly in the center of the coupon with the Allen wrench used to loosen the set screw.

Note: Upon transfer, avoid contact of the coupon with the tube or splashguard. If contact occurs, replace the coupon and associated tube and/or splashguard.
- 13.7 Remove an appropriate number of coupons for testing. Coupons deposited in conical tubes should be used within 30 minutes. Obtain a set of five coupons for test substance treatment and three coupons for the controls.

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Note: To ensure that the maximum biofilm surface area is in contact with the test substance, it is recommended that the coupon sit approximately vertically or at an angle in the bottom of the conical tube following deposit. A flat-lying carrier can cause a difficult-to-remove air bubble under the carrier following addition of the test substance which can impede the ability of the test substance to fully cover the carrier.

- 13.8 After depositing coupons, gently remove the splashguard from each tube and cap the tube to mitigate dehydration.
- 13.9 After removing the appropriate number of coupons, slowly pipette 4 mL of prepared and equilibrated test substance (treatment) or PBDW (untreated control) down the side of the conical tubes containing the coupons, avoiding direct contact with the coupon during application and being careful to completely cover the coupon.
 - 13.9.1 Process coupons treated with test substance first, followed by controls.
 - 13.9.2 For a ten minute contact time, a 30 second or one minute interval between coupons is recommended.
- 13.10 Immediately after addition of test substance or PBDW (for controls), gently swirl each tube 1 – 2 times to fully expose the biofilm on the coupon to liquid, ensuring there are no air bubbles trapped beneath the coupon. Do not shake the tubes. If following the gentle swirl an air bubble still remains under or around the carrier, the carrier may be discarded, and a new carrier may be tested. For those test substances that cause effervescence, the presence of the effervescence does not invalidate the coupon.
- 13.11 Allow the tubes to remain at room temperature (e.g. $21 \pm 2^{\circ}\text{C}$), or other specified temperature, for the duration of the contact time. A water bath may be used, if necessary.
- 13.12 At the end of the contact time, add the appropriate volume of neutralizer to each tube. Replace the cap and briefly vortex the tube. This represents the 10^0 dilution tube.
 - 13.12.1 For test and control carriers exposed in 50 mL conical tubes, 36 mL of neutralizer will be applied.

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- 13.13.2 For test carriers exposed in 250 mL conical tubes, 196 mL of neutralizer will be applied. Some test substances (e.g. highly acidic products or products with a high peroxide concentration) need additional neutralizer. When 196 mL of neutralizer is used, it is best to pre-aliquot out the neutralizer prior to initiating the exposure time. This volume of neutralizer can be quickly poured into the 250 mL conical treatment tube instead of pipetting.
- 13.13 Following neutralization, remove and disaggregate the biofilm from the treated and control coupons.
- 13.13.1 Vortex each tube on the highest setting, ensuring a complete vortex, for 30 ± 5 seconds.
- 13.13.2 After the first vortex, suspend the tubes in an ultrasonic water bath so that the liquid level in the tubes is even with the water level in the tank of the bath. Do not allow the tubes to touch the bottom or sides of the ultrasonic water bath. Ensure that the water bath has been previously degassed for approximately five minutes.
- 13.13.3 Sonicate the tubes at 45 ± 5 kHz for 30 ± 5 seconds.
- 13.13.4 Vortex the tubes as listed in 13.13.1.
- 13.13.5 Sonicate the tubes as listed in 13.13.3.
- 13.13.6 Vortex the tubes as listed in 13.13.1. These tubes are considered the 10^0 dilution.
- 13.14 Dilute and recover disaggregated biofilm samples.
- 13.14.1 Serially dilute the disaggregated biofilm samples from treated and control coupons ten-fold in PBDW.
- 13.14.2 For treated coupons, filter at least 25% of the total volume of neutralizer + test substance from the 10^0 dilution tube and the entire contents of the 10^{-1} and 10^{-2} dilution tubes (9 mL and 10 mL, respectively) through a $0.45\mu\text{m}$ filter. Additional dilutions may be performed for test carriers, where appropriate, and the entire contents filter plated as directed below.

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- 13.14.2.1 Prior to filtering, pre-wet the filter membrane with approximately 20 mL of PBDW.
- 13.14.2.2 Vortex the treatment or dilution tube prior to filtration or plating.
- 13.14.2.3 For 40 mL neutralized volumes, filter at least 10 mL from the 10^0 dilution tube.
- 13.14.2.4 For 200 mL neutralized volumes, filter at least 50 mL from the 10^0 dilution tube.
- 13.14.2.5 If filtering the entire contents of a dilution tube, rinse the tube with approximately 10 mL of PBDW and filter the rinsate in the same filter.
- 13.14.2.6 Pass liquid from the 10^0 dilution tube through the filter within approximately 1 minute following initiation of filtering. Ensure limited pooling of liquid on the filter apparatus – use multiple filters per sample if necessary (i.e. slow filtration due to large volumes of neutralizer).
- 13.14.3 Rinse the sides of the filter funnel with approximately 40 mL of PBDW, evacuate and transfer the membrane filter to R2A (for *P. aeruginosa*) or BHI agar (for *L. monocytogenes*). Gently roll the filter onto the surface of the agar to prevent trapping air bubbles between agar and the membrane.
- 13.14.4 For control coupons, spread plate 0.1 mL aliquots of the appropriate dilutions (e.g. 10^{-4} – 10^{-6} for *P. aeruginosa* or 10^{-2} – 10^{-4} for *L. monocytogenes*) in duplicate onto R2A (for *P. aeruginosa*) or BHI agar (for *L. monocytogenes*).
- 13.15 Incubate control plates at $35 \pm 2^\circ\text{C}$ for 48 ± 4 hours. Incubate test plates at $35 \pm 2^\circ\text{C}$ for 72 ± 4 hours. If necessary, following incubation all plates may be stored at $2 - 8^\circ\text{C}$ for up to three days prior to examination.

14.0 CONTROLS

14.1 Neutralization Confirmation Control

- 14.1.1 May be performed prior to or concurrent with the test.

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- 14.1.2 Defrost a single cryovial and briefly vortex to mix. For *P. aeruginosa*, add 10 µL of the thawed frozen stock to a tube containing 10 mL of TSB (30 g/L) and vortex to mix. For *L. monocytogenes*, add 10 µL of the thawed frozen stock to a tube containing 10 mL of BHI (37 g/L) and vortex to mix. Incubate the bacterial suspension at $35 \pm 2^\circ\text{C}$ for 24 ± 2 hours.
- 14.1.3 Prepare ten-fold serial dilutions in PBDW to achieve concentrations of approximately 10^4 and 10^3 CFU/mL per dilution tube (these are typically observed in the 10^{-5} and 10^{-6} dilution tubes, respectively). Alternate dilutions may be used where applicable. When diluted and plated, at least one dilution should result in counts of 20 – 200 CFU/filter.
- 14.1.4 Neutralization Confirmation Treatment (Test A)
- 14.1.4.1 At timed intervals, add 4 mL of test substance use-solution to 36 mL of neutralizer (or 196 mL if increased volume is to be used in test) and briefly vortex mix. Within approximately 10 seconds, add 0.1 mL of diluted test organism and vortex mix. Perform in triplicate for each organism and dilution used. Proceed to 14.1.7.
- 14.1.5 Neutralizer Toxicity Treatment (Test B)
- 14.1.5.1 At timed intervals, add 0.1 mL of the diluted test organism to 40 mL of neutralizer (or 200 mL if increased neutralizer volume is to be used in test) and vortex mix well. Perform in triplicate for each organism and dilution used. Proceed to 14.1.7.
- 14.1.6 Test Culture Titer (Test C)
- 14.1.6.1 At timed intervals, add 0.1 mL of the diluted test organism to 40 mL of PBDW (or 200 mL if increased neutralizer volume is to be used in test) and vortex mix well. Perform in triplicate for each organism and dilution used. Proceed to 14.1.7.
- 14.1.7 Hold all treatments at room temperature (e.g. $21 \pm 2^\circ\text{C}$) for 10 minutes \pm 30 seconds.

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14.1.8 Following the holding period, vortex mix each treatment tube and filter a minimum of 25% of the total volume of neutralizer + test substance (i.e. 10 mL if total neutralized volume = 40 mL, or 50 mL if total neutralized volume = 200 mL) through 0.45 µm membrane filter pre-wetted with approximately 20 mL of PBDW. If necessary, multiple filters may be used to filter larger volumes. Use the same filtration volume for Test A, B and C.

14.1.9 Rinse the sides of the filter funnel with additional PBDW and transfer the membrane filter to R2A (for *P. aeruginosa*) or BHI agar (for *L. monocytogenes*). Gently roll the filter onto the surface of the agar to prevent trapping air bubbles between agar and the membrane.

14.1.10 Incubate control plates at a temperature and time as performed in the test. Count colonies per filter. If the filtered volume was split over multiple filters, add the counts together.

14.1.11 Test C must demonstrate approximately 20 – 200 CFU/filter. Neutralization is confirmed (and valid) when the recovered CFU in Test A and Test B is within 50% of the Test C recovered CFU.

Note: Test A and Test B CFU counts higher than Test C are deemed valid.

14.2 Test Substance Diluent Sterility Control

14.2.1 Spread plate 1 mL of the test substance diluent onto an appropriate agar. No growth is required for a valid control.

14.3 Continuous Flow Operation Media Sterility Control

14.3.1 Spread plate 1 mL of the prepared Continuous Flow Operation medium onto an appropriate agar. No growth is required for a valid control.

14.4 Neutralizer Sterility Control (if applicable)

14.4.1 If not performed as part of routine media preparation quality control (i.e. preparation of D/E + 0.01% catalase on the day of test), spread plate 1 mL of the neutralizer onto an appropriate agar. No growth is required for a valid control.

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- 14.5 Incubate Sterility and Neutralization Confirmation control plates at $35 \pm 2^\circ\text{C}$ for 48 ± 4 hours. If necessary, following incubation plates may be stored at $2 - 8^\circ\text{C}$ for up to three days prior to examination.
- 14.6 Purity Control
- 14.6.1 Test organism purity is confirmed prior to efficacy testing. See 9.4 for procedure. If purity of test organism cannot be confirmed, biofilm growth may be stopped prior to use of carriers in efficacy testing.

15.0 RECORDING RESULTS & CALCULATIONS

- 15.1 Count the number of colonies. Counts in excess of 200 CFU per filter or 300 CFU for spread plating should be recorded as Too Numerous To Count (TNTC). If no colonies are present, record as zero.
- 15.2 Inspect the growth on the filters and plates for purity and typical characteristics of the test organism. Gram stain one representative colony per coupon set with growth for treated and controls.
- 15.2.1 *P. aeruginosa* (ATCC 15442): Gram negative rod. It may display three colony types: a) circular, undulate edge, convex, rough and opaque; b) circular, entire edge, convex, smooth and translucent; c) irregular, undulate edge, convex, rough, spreading and translucent. A vibrant green/yellow-green color may be present on R2A agar.
- 15.2.2 *L. monocytogenes* (ATCC 49594): Gram positive rod. Small to medium circular, smooth, white or cream-colored colonies.
- 15.3 The CFUs at each dilution are recorded and used to calculate the log reduction.
- 15.4 Calculate the CFU/coupon for control coupons; use the following equation:

$$\left[\frac{\left(\frac{\text{Mean CFU for } 10^W + \text{Mean CFU for } 10^X}{10^W + 10^X} \right)}{Y} \right] \times Z$$

Where:

10^W and 10^X are the dilution tubes plated.

Y is the volume plated (mL).

Z is the volume of liquid (PBDW + neutralizer) in the tube with the coupon.

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- 15.5 Calculate the CFU/coupon for treated coupons; use the following equation:

$$\left[\frac{\text{CFU per filter for } 10^w + \text{CFU per filter for } 10^x}{(a \times 10^w) + (b \times 10^x)} \right] \times Z$$

Where:

10^w and 10^x are the dilution tubes plated.

“a” and “b” are the volumes filtered at each dilution.

Z is the volume of liquid (test substance + neutralizer) in the tube with the coupon.

- 15.6 Calculate the \log_{10} density of the CFU/coupon for each treated and control coupon.
- 15.7 Calculate the mean \log_{10} density (LD) across all treated and control coupons. For a valid test, the mean *P. aeruginosa* control LD must be between 8.0 and 9.5 \log_{10} with each coupon exhibiting a log density of 8.0 – 9.5. For a valid test, the mean *L. monocytogenes* control LD must be between 6.5 and 8.0 \log_{10} with each coupon exhibiting a log density of 6.5 – 8.0.

$$\text{Mean LD} = \frac{\text{LD of Coupon \#1} + \text{LD of Coupon \#2} \dots + \text{LD of Coupon \#n}}{\text{Total number of carriers per set}}$$

Where:

n is the total number of coupons tested (i.e. 3 for control set, 5 for test set).

- 15.8 Calculate the \log_{10} reduction (LR) for the treated coupons.

$$\text{LR} = \text{Mean Control coupon LD} - \text{mean Treated coupon LD}$$

- 15.9 If there is no recovery of survivors for the treated coupons and the entire contents of the 10^0 tube are filtered, the LR is greater than or equal to the mean control counts.
- 15.10 If there is no recovery of survivors for the treated coupons and a fraction of the volume from the 10^0 tube is filtered, substitute < 1 CFU at the 10^0 dilution in the equation in 15.5.

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16.0 PERFORMANCE STANDARD

16.1 A claim of efficacy for the control of *Pseudomonas aeruginosa* and *Listeria monocytogenes* biofilm on food contact surfaces may be added to an EPA registered antimicrobial pesticide product that meets the following test criteria:

- Test 3 batches of test substance at the Lower Certified Limit against the biofilm of both *P. aeruginosa* and *L. monocytogenes* on separate test dates (*P. aeruginosa* and *L. monocytogenes* tests may be conducted on the same day).
- For *P. aeruginosa*, a minimum average 6 log reduction should be obtained, testing 5 coupons per batch.
- For *L. monocytogenes*, a minimum average 5 log reduction should be obtained, testing five coupons per batch.
- The mean log density for *P. aeruginosa* control coupons (CFU/coupon) should be 8.0 – 9.5 log with each coupon exhibiting a log density of 8.0 – 9.5 log.
- The mean log density for *L. monocytogenes* control coupons (CFU/coupon) should be 6.5 – 8.0 log with each coupon exhibiting a log density of 6.5 – 8.0 log.
- If the control coupon mean log density is above the required upper range value and passing test results are achieved, testing is considered valid and does not need to be repeated. If the control mean log density is below the lower range value and testing results in a failure, testing may not be repeated. If testing results in a failure and the control coupon mean log density is above the accepted range, testing may be repeated.

17.0 RELATED FORMS

- 17.1 Form 3170: CDC Biofilm Reactor Carrier Processing Record
- 17.2 Form 3190: Determination of Antimicrobial Product Efficacy Against Food Contact Surface Associated Biofilm
- 17.3 Form 3191: Shared Generation of Food Contact Surface Associated Biofilm

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18.0 REFERENCES

- 18.1 MS008: Synthetic Hard Water Preparation & Standardization
- 18.2 MS032: Daily Operations – Preservation of Microbial Cells
- 18.3 MS102 Attachment
- 18.4 ASTM E2562-17: Standard Test Method for Quantification of *Pseudomonas aeruginosa* Biofilm Grown with High Shear and Continuous Flow Using CDC Biofilm Reactor
- 18.5 ASTM E2871-19: Standard Test Method for Evaluating Disinfectant Efficacy Against *Pseudomonas aeruginosa* Biofilm Grown in CDC Biofilm Reactor Using Single Tube Method
- 18.6 ASTM E3161-18: Standard Practice for Preparing a *Pseudomonas aeruginosa* or *Staphylococcus aureus* Biofilm using the CDC Biofilm Reactor
- 18.7 US EPA Office of Pesticide Programs Standard Operating Procedure MB-19: Growing a Biofilm Using the CDC Biofilm Reactor – May 2017
- 18.8 US EPA Office of Pesticide Programs Standard Operating Procedure MB-20: Single Tube Method for Determining the Efficacy of Disinfectants Against Bacterial Biofilm – May 2017

19.0 MOST RECENT REVISION SUMMARY

New Standard Operating Procedure.

MS102 Attachment

CDC Biofilm Reactor Number	Reactor Operating Volume*
#1	316 mL
#2	332 mL
#3	323 mL
#4	340 mL
#5	332 mL

* CDC Biofilm Reactor operating volumes were determined as described in MS102. Operating volumes were confirmed to be accurate at both 60 ± 5 RPM and 125 ± 5 RPM.

Form-Ver. 6016-09
Effective: 05/01/18
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Regulated Study Protocol Amendment

Study Title: Synergex Efficacy against Food Contact Surface Associated Biofilm
Study Number: 1900080
Amendment Number: 1900080-1A
Amendment Effective Date: 8/29/19

Description of Amendment

On Page 6 of 11 in the Study Protocol, the 810.2200 guideline referenced is corrected to be titled: U.S. EPA Office of Chemical Safety and Pollution Prevention Product Performance Test Guidelines 810:2200 – Disinfectants for Use on Environmental Surfaces – Guidance for Efficacy Testing (February 2018).

Scientific Basis for Amendment

The word "Test" was inadvertently missing from the guideline title as originally listed in the protocol. The protocol is amended to appropriately correct the name.

- ☒ This amendment does not affect the integrity of the study.
☐ This amendment does affect the integrity of the study.

☐ This protocol amendment has been amended.

Refer to protocol amendment _____ for details.

Initial & Date _____

Study Director

8.29.19
Date